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Interaction of Operational and Physicochemical Factors Leading to *Gordonia amarae*-Like Foaming in an Incompletely Nitrifying Activated Sludge Plant

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The overgrowth of *Gordonia amarae*-like bacteria in the mixed liquor of an incompletely nitrifying water reclamation plant was inversely correlated with temperature ($r = -0.78$; $P < 0.005$) and positively correlated with the solids retention time (SRT) obtained a week prior to sampling ($r = 0.67$; $P < 0.005$). Drops followed by spikes in the food-to-mass ratio (0.18 to 0.52) and biochemical oxygen demand concentrations in primary effluent (94 to 298 mg liter^{-1}) occurred at the initiation of *G. amarae*-like bacterial growth. The total bacterial concentration did not increase as concentrations of *G. amarae*-like cells increased, but total bacterial cell concentrations fluctuated in a manner similar to that of *G. amarae*-like bacteria in the pseudo-steady state. The ammonium ion removal rate (percent) was inversely related to *G. amarae*-like cell concentrations during accelerated growth and washout phases. The dissolved oxygen concentration decreased as the *G. amarae*-like cell concentration decreased. The concentrations of *G. amarae*-like cells peaked (2.47×10^9 cells liter^{-1}) approximately 1.5 months prior to foaming. Foaming occurred during the late pseudo-steady-state phase, when temperature declines reversed. These findings suggested that temperature changes triggered operational and physicochemical changes favorable to the growth of *G. amarae*-like bacteria. Fine-scale quantitative PCR (qPCR) monitoring at weekly intervals allowed a better understanding of the factors affecting this organism and indicated that frequent sampling was required to obtain statistical significance with factors changing as the concentrations of this organism increased. Furthermore, the early identification of *G. amarae*-like cells when they are confined to mixed liquor (10^7 cells liter^{-1}) allows management strategies to prevent foaming.

Foaming incidents in aeration tanks have been reported worldwide since 1969 (3), yet little progress in early diagnosis and prevention has been made. Additionally, foam causes considerable operational difficulties, resulting in problems with the separation of solids. Filamentous bacteria such as nocardioforms and "*Candidatus* Microthrix parvicella" are commonly identified as the cause of foaming events (37, 51, 55). The foaming capability of these Gram-positive organisms is related to the strongly hydrophobic chemical nature of the cell wall and the extracellular excretion of biosurfactants involved in foam initiation and stability (28, 33, 47). Previous studies using either sewage or pure cultures showed that hydrophobic compounds or surfactants from commercial or household products also enhanced foaming (6, 28). Although growth medium harvested from cultures of *Gordonia amarae* generated foam, there was less foam than when organisms were present (28).

Surveys of foaming events indicated certain crucial shared features. First, the mechanisms promoting foaming span a variety of biological treatment processes (17, 61, 70). Second, the vast majority of treatment plants are affected by foaming problems. For example, 88% of 47 plants in North Carolina and 97% of 31 plants in Illinois experienced foaming (16, 35). Only 8% of 46 plants studied in Queensland, Australia, never reported occurrences of foaming (9). This illustrates the lack of an understanding of the physical, chemical, and biological factors that initiate and promote foaming events across treatment processes as well as the difficulty in eliminating foaming on a permanent basis.

A number of operational and physicochemical parameters, such as extended mean cell residence times (MCRTs), high concentrations of long-chain hydrocarbons, certain pH ranges, and temperatures, were found to be factors that promoted foaming events (9, 10, 43, 51). However, no common operational parameters, design criteria, and sewage compositions were found to be predictive of foaming events in full-scale Australian wastewater treatment plants (9). Conflicting reports make it difficult to forecast the occurrence of this group of organisms. Furthermore, the use of light microscopy results in late identification (after filaments have formed), making the prevention of foaming events more difficult (38). These findings emphasized the problem with clustering a wide variety of filamentous foaming bacteria into one group such as nocardioforms by using light microscopy, and later research revealed that nocardioform organisms were actually different genera within the suborder *Corynebacterineae* (57, 58, 62). Unfortunately, even today, most treatment plants use light microscopy to identify foaming bacteria, which often results in errors in identification (66), making causal comparisons of plant conditions difficult.

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The application of molecular methods to the identification of foaming bacteria has several advantages over light microscopy, because the latter differentiates filamentous bacteria based on variations in morphology (38). Nucleic acid tools identify organisms through the recognition of specific nucleic acid base designations and, if combined with a fluorescent probe, can quantify accurately the copy numbers of the nucleic acid target (25). Molecular techniques provide far greater specificities in the identification of filamentous bacteria and have demonstrated what proportion of the population is metabolically active using rRNA (25, 45). However, these techniques are expensive and time-consuming and require extensive training. Molecular procedures such as quantitative PCR (qPCR) have been used to monitor type 021N organisms, bulking bacteria, and "*Candidatus* Microthrix parvicella," foaming bacteria, in sewage treatment plant samples (34, 39, 65). *Gordonia* spp. have been identified as the causative agent of most foaming events reported in the literature, and their abundance has been assessed by using light microscopy, membrane hybridization, or fluorescent *in situ* hybridization (FISH) (15, 17, 18, 51). Most recently, PCR-reverse line blot hybridization (PCR-RLB) has allowed the simultaneous identification of several foaming filamentous bacteria (4).

We report here the first qPCR study which monitored *G. amarae*-like cell concentrations in the mixed liquor of a full-scale, incompletely nitrifying plant to elucidate the interaction of *G. amarae*-like bacteria with physicochemical and operational factors that led to a foaming incident. Moreover, this study delineated that the initiation of *G. amarae*-like bacterial growth corresponded to (i) seasonal temperature shifts, (ii) an increased solids retention time (SRT), and (iii) sudden drops in the food-to-mass (F/M) ratio and biochemical oxygen demand (BOD) concentration in primary effluent followed immediately by increases in both of these parameters. However, these parameters were not associated with the foaming event. Thorough fine-scale monitoring of *G. amarae*-like bacterial population dynamics before, during, and after the foaming incident is described. These findings suggested that this organism's occurrence can be identified long before foaming occurs, when the cell length is short and unbranched (14) and cells are confined to the mixed liquor, which corresponded to a concentration of 10^7 cells liter^{-1}. Identification at this early growth stage, before foaming begins, would increase the opportunity to eliminate this filamentous bacterium through the manipulation of the SRT or the chlorination of return activated sludge (RAS).

MATERIALS AND METHODS

Pure cultures, culture media, and growth conditions. Twenty-two bacterial strains were provided by the Agricultural Research Service (USDA, IL), including *Bacillus atrophaeus* (B-363), *Corynebacterium glutamicum* (strain B-2784), *Enterobacter cloacae* (B-23267), *Dietzia maris* (B-16841), *Gordonia amarae* (B-8176), *Gordonia lacunae* (B-24551), *Gordonia sihwensis* (B-24155), *Gordonia sputi* (B-16936), *Gordonia terrae* (B-16283), *Microbacterium barkeri* (B-24231), *Millisia brevis* (B-24424), *Mycobacterium pyrenivorans* (B-24349), *Nocardia brevicatena* (B-2896), *Nocardia flavorosea* (B-16176), *Nocardia carnea* (B-1336), *Nocardia jejuensis* (B-24430), *Nocardia salmonicolor* subsp. *aurantiaca* (B-16249), *Rhodococcus corynebacteroides* (former *Nocardia corynebacteroides*) (B-24037), *Rhodococcus erythropolis* (B-16431), *Streptomyces gardneri* (B-5616), *Tsukamurella sunchonensis* (B-24668), and *Tsukamurella paurometabola* (B-16960). All cultures were grown in medium recommended by the USDA, which was composed of 0.5% tryptone, 0.5% yeast extract, 0.1% glucose, and 0.1% K_2HPO_4 at pH 7.0 (TYG broth). Cultures were incubated in an orbital shaker (Forma Scientific, Marietta, OH) at 28°C at 90 rpm for 48 to 72 h or until growth was observed. For colony isolation, liquid cultures were streaked onto 2% TYG agar and incubated at 30°C for 48 to 72 h or until colonies were observed in an incubator (Precision Scientific, Chicago, IL). An individual colony was picked, transferred into a new tube containing TYG broth, and incubated in an orbital shaker (Forma Scientific, Marietta, OH), as described above. Extracted *Mycobacterium bovis* (ATCC 19210) DNA was obtained from the U.S. Food and Drug Administration Pacific Regional Laboratory Southwest (FDA PRL-SW), Irvine, CA.

The *Escherichia coli* O157:H7 (ATCC 43895) culture was also provided by the FDA PRL-SW, Irvine, CA. *E. coli* cells were cultured as described previously (24).

Wastewater sample collection. Wastewater samples were collected by using a portable pump (American Sigma, Loveland, CO) from the middle of the aeration tank in 250-ml sterile containers on a weekly basis from 15 August 2008 to 5 August 2009, totaling 48 samples from the Chiquita Water Reclamation Plant (CWRP). An additional foam sample was collected on 18 March 2009 from the CWRP. Mixed liquor from the Oso Water Reclamation Plant (OWRP) was taken once in 250-ml sterile containers on 30 December 2008. All samples were kept on ice, transported to the laboratory, and refrigerated at 4°C until they were analyzed, within 24 h.

Operational parameters of the CWRP and OWRP are presented as means ± standard deviations. The 6-million-gallon-per-day (6-MGD) Chiquita Water Reclamation Plant had a dissolved oxygen (DO) concentration of 0.87 ± 0.16 mg liter^{-1}, a hydraulic retention time (HRT) of 4.4 ± 0.2 h, and an SRT of 4.3 ± 0.5 days. The 2 MGD Oso Water Reclamation Plant had a DO concentration of 1.84 ± 0.67 mg liter^{-1}, an HRT of 6.25 ± 0.24 h, and an SRT of 2.8 ± 0.4 days. Both plants incompletely nitrify wastewater.

DNA extraction. DNA was extracted from 1-ml pure cultures and environmental samples by using a modified bead-beating protocol (67, 71), according to methods described previously by Huang et al. (29). DNA was extracted from three subsamples of each sample. DNA was diluted 1:10 to determine the concentration and purity by using a DU7400 spectrophotometer (Beckman, Orange, CA). The purity (A_{260}/A_{280}) ranged between 1.60 and 1.80. Sterile high-performance liquid chromatography (HPLC)-grade water (Fisher Scientific, Fairlawn, NJ) was used to dilute pure culture extracts and environmental extracts into two sets of 1:50 and 1:100 dilutions for future qPCR analyses. The foam extract was diluted 1:1,000. The diluted and undiluted DNA samples were immediately stored at −50°C and −80°C, respectively, until use.

Design of *Gordonia amarae* primers and probes. The *Gordonia amarae* primers and probes were constructed in three parts. The first step determined the regions of homology and heterogeneity in partial sequences of 16 rRNA genes among *G. amarae* strains. For this step, nine 16S rRNA gene sequences for *Gordonia amarae* (GenBank accession numbers AF020331, AF020330, X82243, X75902, X80635, NR_037032, AF020332, AF020329, and X80601) available in the GenBank database (http://www.ncbi.nlm.nih.gov/) were aligned by using the Basic Local Alignment Search Tool (BLAST/blastn Suite) to form 5′-to-3′ consensus sequences (1). In the second step, a 16S rRNA gene of *G. amarae* (accession number AF020331) was compared to and aligned with those of all other bacteria by using the Basic Local Alignment Search Tool, excluding uncultured/environmental sample sequences (BLAST/blastn Suite), to form 5′-to-3′ consensus sequences and to access regions of high dissimilarity compared to the *G. amarae* 16S rRNA gene consensus sequence (accession number AF020331) (1). A primer and probe set, designated G-amarae16S-F, G-amarae16S-R, and G-amarae-Probe (Table 1), was selected, which had the least homology to other *Gordonia* spp. and all other bacterial species but shared homology to all *G. amarae* 16S rRNA gene sequences. In the third step, sequences of primers G-amarae16S-F and -R and probe G-amarae-Probe were then rescreened by using BLASTN (1) to

TABLE 1 16S rRNA gene primer and probe sets for bacteria examined in this study[d]

Primer or probe	Target bacterium(a)	Sequence (5′–3′)[e]	T_m (°C)	Nucleotide positions	Reference
Primers					
G-amarae16S-F	*G. amarae*	AAAGGCCCCTTCGGGGGTAC	70.8	53–72[a]	This study
G-amarae16S-R	*G. amarae*	GGCCCATCCCTGACCGCAAA	75.3	211–192[a]	This study
N-spp-Myc-F1	Mycolata	CTGGGCGTAAAGAGCTTGTA	61.9	539–558[b]	5
Mycolata-1004r	Mycolata	GCCATGCACCACCTGTACAC	66.5	1014–1033[b]	5
1055f	Total bacteria	ATGGCTGTCGTCAGCT	57.7	1055–1070[c]	40
1392r	Total bacteria	ACGGGCGGTGTGTAC	58.9	1406–1392[c]	22
Probes					
G-amarae-Probe	*G. amarae*	FAM-ACCTGCTCCTGCATGGGGGTGGG-BHQ1	79.3	160–182[a]	This study
16STaq1115	Total bacteria	FAM-CAACGAGCGCAACCC-BHQ1	62.9	1100–1114[c]	27

[a] Based on data under GenBank accession number AF020331.
[b] Based on data under GenBank accession number AP006618.
[c] Based on data under GenBank accession number AP001368.
[d] All primers and probes were developed based on the 16S rRNA gene.
[e] FAM, 6-carboxyfluorescein; BHQ1, black hole quencher 1.

determine cross-reactivity. Primers G-amarae16S-F and G-amarae16S-R yielded 159-bp amplicons. Although there were some genera which had a nucleic acid sequence that matched most of the forward or reverse primers, none of them produced an amplicon of a similar size, or no amplicon was formed because only one primer shared homology. An *in silico* check was performed by using In Silico PCR amplification software (http://insilico.ehu.es/PCR/) (7).

Optimization of PCR conditions for the G-amarae16S primer set. A temperature gradient was performed by using whole-DNA extracts of a *G. amarae* pure culture (B-8176), which indicated that the optimum annealing temperature for the primers was 69.9°C. The optimum concentration of $MgCl_2$ using an annealing temperature of 69.9°C was 4.5 mM, with the concentration increasing in 0.5 mM increments from 2 to 5 mM.

qPCR positive control and standard. DNA standards for *G. amarae* and total bacterial quantifications using qPCR were obtained by using *G. amarae* (B-8176) and *E. coli* O157:H7 (ATCC 43895) pure culture extracts, respectively. Each qPCR standard curve was generated by using ranges of 3.3×10^2 to 3.3×10^5 and 5.0×10^3 to 5.0×10^6 copies per qPCR for *G. amarae* and total bacteria, respectively. Every qPCR run was accompanied by a standard curve, as described above, and a negative control.

PCR and qPCR amplification. Each environmental nucleic acid extract was amplified in triplicate by using qPCR. Primer pair G-amarae16S-F and -R was used to amplify a 159-bp fragment of the *G. amarae* 16S rRNA gene, and gene copies were quantified by using qPCR with a dually labeled fluorescent probe (Table 1). The *G. amarae* qPCR protocol for environmental DNA extracts was 2 min of holding at 94°C, 20 s of denaturation at 94°C, and 40 s of annealing at 69.9°C per cycle for 35 cycles. The master mix was composed of 4.5 mM $MgCl_2$ (TaKaRa, Tokyo, Japan), 1× buffer (TaKaRa, Tokyo, Japan), 200 nM deoxynucleoside triphosphate (dNTP) (TaKaRa, Tokyo, Japan), 0.75 U AmpliTaq DNA polymerase (TaKaRa, Tokyo, Japan), 200 nM each forward primer and reverse primer (Sigma-Genosys, Woodlands, TX), 100 nM probe (Biosearch Technologies, Novato, CA), and 100 ng μl^{-1} of bovine serum albumin (BSA) (New England Biolabs, Ipswich, MA) and was brought to a final volume of 20 μl with sterile HPLC-grade water, to which 5 μl of each DNA sample was added. The DNA sample template concentrations ranged from 2 to 20 ng μl^{-1} in each reaction mixture. The *G. amarae* qPCR was run on an Eppendorf RealPlex EP instrument (Eppendorf, Hauppauge, NY). The minimum detection limit was 33 copies of the *G. amarae* 16S rRNA gene per reaction. The efficiency of all qPCR runs ranged between 0.95 and 1.03, and the coefficient of determination (R^2) ranged between 0.997 and 1.000. The PCR run for *G. amarae* used the same the temperature cycle protocol and master mix described above, but the probe was not added.

Primers 1055f and 1392r (Sigma-Genosys, Woodlands, TX) and probe 16STaq1115 (Biosearch Technologies, Novato, CA) were used to quantify total bacteria (Table 1). This primer set yielded a 353-bp amplicon. The composition of the total bacterial qPCR master mix was the same as that of the *G. amarae* master mix described above, except that final concentrations of 3.5 mM $MgCl_2$, 150 nM probe 16STaq1115, and 200 nM primers 1055f and 1392r were used. The total bacterial qPCR protocol, modified from methods described previously by Harms et al. (27), was 2 min of holding at 95°C, 15 s of denaturation at 95°C, and 45 s of annealing at 50°C for each cycle for a total 45 cycles. All total bacterial reaction mixtures were analyzed on a Rotorgene 3000 instrument (Qiagen, Valencia, CA). The minimum detection limit was 5.0×10^3 copies of the 16S rRNA gene per reaction. The DNA template concentrations of environmental extracts ranged from 1 to 10 ng μl^{-1} in each qPCR mixture. The average efficiency of all total bacterial qPCR runs for total bacteria was 0.95, and the mean coefficient of determination (R^2) was 0.9960.

The Chiquita foam DNA extract was run by using primers N-spp-Myc-F1 and Mycolata-1004r (Sigma-Genosys, Woodlands, TX), which yielded a 496-bp amplicon (Table 1). The PCR mixtures were analyzed on a GeneAmp 2700 PCR system (Applied Biosystems, Foster City, CA), and the PCR protocol and master mix used were described previously by Asvapathanagul (5). The master mix used in this PCR run was the same as that described above for the *G. amarae* qPCR runs, except that a final concentration of 3.5 mM $MgCl_2$ (TaKaRa, Tokyo, Japan) was used, without the addition of a probe. The PCR protocol was modified from that used for the *G. amarae* qPCR run, except that an annealing temperature of 60°C was used.

Melt curves. Sybr green I (Invitrogen, Carlsbad, CA) was added to *G. amarae* PCR master mix at a 0.05× final concentration. Sybr green reactions were performed using an Eppendorf RealPlex EP instrument (Eppendorf, Hauppauge, NY), with the temperature ramped from 55°C to 99°C in 1°C increments.

Analysis of PCR products, PCR product purification, and DNA sequencing. The 159-bp amplicon of primers G-amarae16S-F and -R and the 496-bp amplicon of primers N-spp-Myc-F1 and Mycolata-1004r were subjected to gel electrophoresis. The amplicon size was determined by using 8 μl of PCR products mixed with 3 μl of 6× gel loading buffer type IV (53), loaded onto a 2% (wt/vol) agarose (Fisher Scientific, Fairlawn, NJ) gel soaked with 0.375 μg ml^{-1} ethidium bromide (Fisher Scientific, Fairlawn, NJ). The gel was run at 90 V for 90 min. Two and a half microliters of a 50- or 100-bp DNA miniladder (exACTGene; Fisher Scientific International, Canada) was added to the first and last lanes of the agar. Product bands were photographed by using FluorChem (Alpha Innotech Corporation, San Leandro, CA), and image analysis was performed by using AlphaEaseFC software (Alpha Innotech Corporation, San Leandro,

CA). Bands were excised and purified by using QIAquick gel extraction kits (Qiagen Sciences, Valencia, CA) according to the manufacturer's instructions. The 159-bp amplicons and the 496-bp amplicon were sent to the UC Davis sequencing facility (Davis, CA) for sequencing analysis. DNA sequence determinations were carried out on both 5′-to-3′ and 3′-to-5′ strands to verify the actual nucleic acid composition. The sequencing results were visualized by using Sequence Scanner V1.0 software (Applied Biosystems, Foster City, CA). Fragment alignment was performed by using CLUSTALW software (http://workbench.sdsc.edu/) (63) or the Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/) (1) to yield a consensus sequence for each sample.

Calculation of qPCR DNA standards. The molecular weight of the nucleotide base pair, 660 pg per picomole (54), was used to convert the DNA concentration (ng μl^{-1}) to genomic DNA copy numbers (copies μl^{-1}) for *G. amarae* (B-8176) and *E. coli* (ATCC 43895). The *G. amarae* (B-8176) and *E. coli* (ATCC 43895) genome sizes were based upon those of *Gordonia bronchialis* (GenBank accession number CP001802) and *Escherichia coli* O157:H7 strain EDL933 (accession number NC_002655), respectively (30, 50). Two and seven copies of the 16S rRNA gene per genome of *G. bronchialis* (accession number CP001802) and *Escherichia coli* O157:H7 strain EDL933 (accession number NC_002655) were used as the numbers of 16S rRNA genes per genomic DNA of *G. amarae* (B-8176) and *E. coli* (ATCC 43895), respectively (30, 50).

Calculation of qPCR results. Two and 3.6 copies of the 16S rRNA gene per genome were used to convert 16S rRNA genes per liter to cell concentrations (cells $liter^{-1}$) for *G. amarae*-like bacteria and total bacteria, respectively (30, 36).

Physical and chemical analysis methods. The temperature and DO concentration were measured at the point of sample collection by using a portable DO meter (Hach, Loveland, CO). A 50-ml volume of each sample was filtered by using 1.5-μm glass microfiber filters (Whatman, Piscataway, NJ) to remove suspended solids prior to ammonium ion (NH_4^+-N) measurements. Ammonium ion (NH_4^+-N) concentrations were analyzed by using an ISOTemp ammonia probe (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions. The mixed-liquor suspended solids (MLSS) concentration, solids retention time (SRT), hydraulic retention time (HRT), flow rate, and total BOD concentration were obtained from laboratory personnel and plant operators, in accordance with standard methods (2, 64).

Statistical calculations. To test the correlation between two variables, the Pearson correlation coefficient (r) was used, and where significance (P) was found, the P value accompanies the r value. Statistical significance (P) was calculated by using the T-distribution function (TDIST) of Microsoft Excel 2007 (44) for both tails. For the statistical analysis, a sample size of 20 was used for the period when *G. amarae*-like cell concentrations were above the detection limit, and sample sizes of 7 and 5 were used for analyses of the accelerating growth phase (rapid and slowing) and washout phase, respectively.

RESULTS

Validation of *Gordonia amarae* primer and probe sets. The DNA extract of CWRP foam was amplified by using primers N-spp-Myc-F1 and Mycolata-1004r, which yielded a 496-bp fragment (GenBank accession number JN638564). The sequencing result showed 99.2% similarity to the *G. amarae* sequence (accession number AF020332) available in the GenBank database (Fig. 1). Based on this information, a primer set, G-amarae16S-F and -R (shown in Table 1), was developed to specifically identify *G. amarae*. Melt-curve analyses of this159-bp amplicon generated from nucleic acid extracts of *G. amarae* (B-8176) and environmental samples indicated that the overall melting temperature (T_m) variation was 0.2°C (see Fig. S1 in the supplemental material). The environmental amplicons generated from OWRP mixed liquor, CWRP mixed liquor, and CWRP foam were sequenced, and they all showed a high level of similarity (96.8%) to *G. amarae* (accession number AF020332) (see Fig. S2 in the supplemental material). The results from melting temperature analyses and sequencing indicated that the foaming organisms from the CWRP and OWRP samples were *G. amarae*-like bacteria. None had 100% similarity to *G. amarae* sequences based on data in the GenBank database (http://www.ncbi.nlm.nih.gov/), which was also true for the 496-bp fragment generated (Fig. 1). Additionally, the specificity of primer set G-amarae-16S-F and -R developed in this study was evaluated by using 23 DNA extracts of pure cultures, belonging mainly to the mycolata group. The results showed that only the *G. amarae* DNA extract (B-8176) produced a 159-bp amplicon after gel electrophoresis (see Fig. S3 in the supplemental material).

Occurrence of *Gordonia amarae*-like cell and total bacterial concentrations. *G. amarae*-like bacteria were observed by using qPCR from 9 December 2008 through 12 May 2009. The *G. amarae*-like cell concentrations varied between 2.30×10^7 and 2.47×10^9 cells $liter^{-1}$ during this time period, but no foam was observed through late February (Fig. 2). Total bacterial concentrations ranged from 1.56×10^{13} to 1.24×10^{14} cells $liter^{-1}$ over this period (Fig. 2). The *G. amarae*-like bacterial population increased rapidly from 9 December 2008 (4.13×10^7 cells $liter^{-1}$) until 7 January 2009 (1.86×10^9 cells $liter^{-1}$), but the total bacterial concentrations fluctuated and did not increase in a similar manner at this time (Fig. 2). In the aeration tank, from 14 January to 12 May 2009, the changes in *G. amarae*-like cell concentrations fluctuated in a way similar to the fluctuations of total bacterial concentrations (Fig. 2). By 11 March 2009, foam was observed to cover 50% of aeration tanks and 100% of the aeration tanks' surfaces from 18 March through 7 April 2009. The maximum *G. amarae*-like cell concentration in the mixed liquor was observed approximately 40 days before the aeration tank surface was covered with foam. Plant personnel did a microscopic examination on 18 March 2009 and found nocardioforms in the foam (Amber Baylor, CWRP laboratory, personal communication).

Decreases of the SRT, water spray, and RAS chlorination were the strategies used to control and eliminate the foaming event. The RAS was chlorinated to control the foam from 31 March to 7 April 2009, but between 7 and 14 April 2009, only the SRT was decreased. Water spraying from 14 through 21 April 2009 and decreased SRT were the treatments used to destroy the foam layer on the surface of the aeration tank. As a result, the *G. amarae*-like cells in foam were combined with *G. amarae*-like cells in mixed liquor, which led to an increased concentration of *G. amarae*-like bacterial cells on 14 April 2009. The RAS chlorination began again between 21 and 28 April 2009 and resulted in a decrease in the *G. amarae*-like cell concentration (Fig. 2).

Effect of SRT on *G. amarae*-like cell concentrations in mixed liquor. The average SRT at the CWRP was 4.3 ± 0.5 days from August 2008 to August 2009 (Fig. 2). During the time period in which *G. amarae*-like bacteria were detected, the SRT averaged 4.2 ± 0.6 days, which was statistically insignificant compared to the SRT over the whole study period. An examination of SRTs before and during the rapid growth period for *G. amarae*-like bacteria (2 December 2008 through 7 January 2009) showed that the SRT increased considerably, from 4.2 to 5.7 days (Fig. 2). The Pearson correlation coefficient between SRTs and *G. amarae*-like cell concentrations over the time period (9 December 2008 to 12 May 2009) was insignificant ($r = 0.04$). However, when the *G. amarae*-like bacterial concentrations were correlated with the SRT

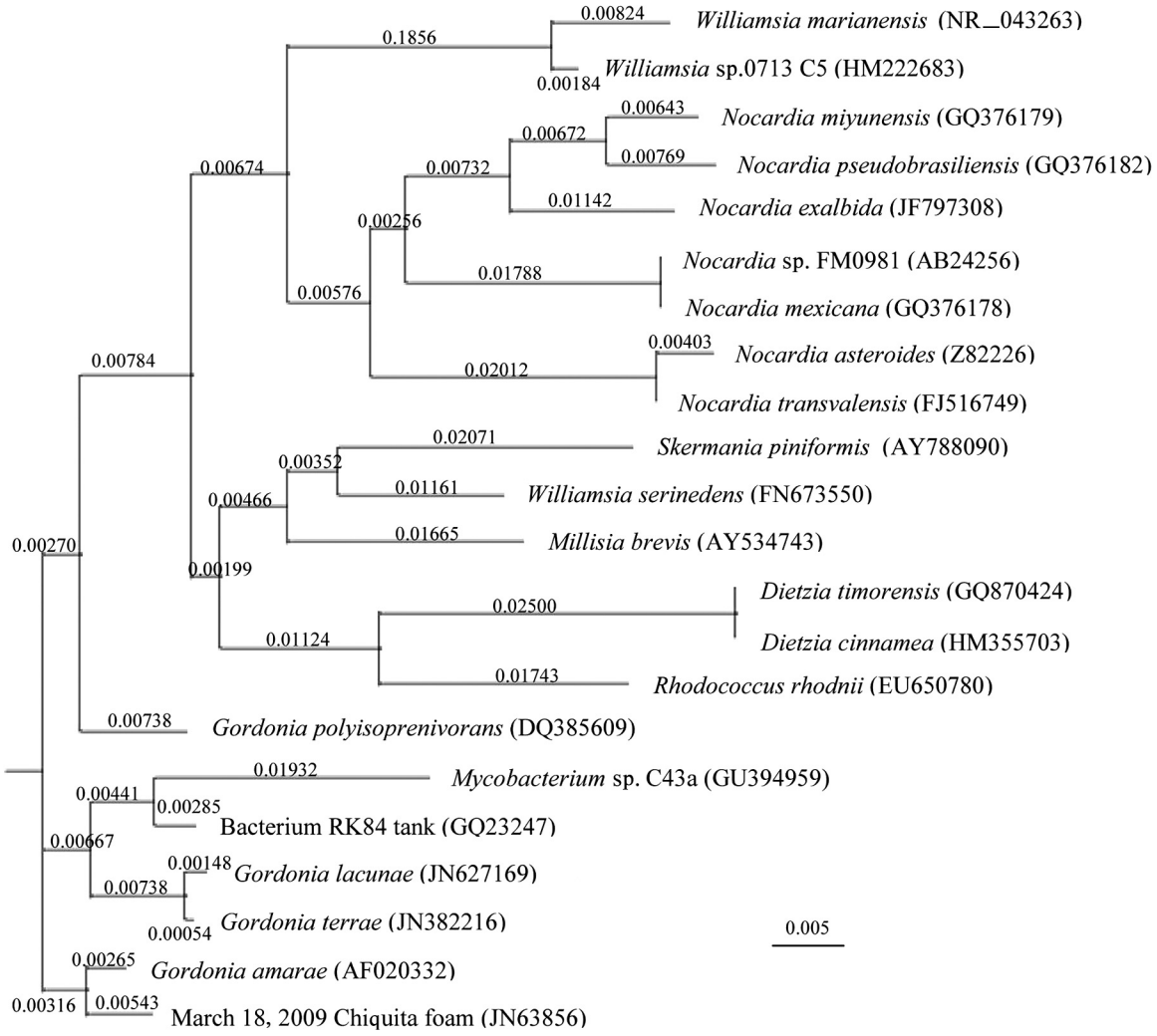

FIG 1 Phylogenetic analysis of the 16S rRNA gene sequences of the 496-bp Chiquita foam sequencing result from 18 March 2009 compared to other acid-fast bacterial 16S rRNA genes. The numbers above the branches indicate the dissimilarity among the sequences. GenBank accession numbers are in parentheses. The scale bar indicates 0.005 inferred 5 nucleic acid base pair differences in 1,000 nucleic acid base pairs.

obtained a week prior to bacterial sampling (lagged SRT), the Pearson correlation coefficient improved to an r value of 0.67 with a P value of <0.005 for the same period. However, total bacterial concentrations showed no correlation with the SRT. The SRT was decreased from the beginning of January until the middle of February, which corresponded to a drop in total bacterial cell concentrations during January only and *G. amarae*-like cell concentrations beginning in February (Fig. 2). Our study found that longer SRTs resulted in higher *G. amarae*-like cell concentrations in mixed liquor, but the longer SRTs were not associated with foam production in the aeration tanks. The SRT was reduced 0.5 days during the week of 7 to 14 April and another 0.5 days during 14 to 21 April 2009 to enhance foam control in the aeration tanks (Fig. 2). Once the concentration of this filamentous bacterium had reached a plateau (pseudo-steady state), the lowering of the SRT to 3.6 days was insufficient to wash out this organism (Fig. 2) in the temperature range of 23.4°C to 24.3°C (Fig. 3a).

Effect of mixed liquor temperature on *Gordonia amarae*-like cell concentrations. The mixed liquor temperature ranged between 23°C and 29°C in the aeration tank over the year period, but the occurrence of *G. amarae*-like bacteria at the CWRP was measurable only when the temperature was ≤26.3°C (Fig. 3a). The initial growth of *G. amarae*-like bacteria started after 2 December 2008 and entered the pseudo-steady state on 28 January 2009, which corresponded to a temperature decrease of 2.9°C. An inverse relationship between *G. amarae*-like cell concentrations (log) and temperature from 9 December 2008 to 12 May 2009 was demonstrated by using Pearson correlation analysis ($r = -0.78$; $P < 0.005$). The total bacterial concentrations were also negatively correlated with temperature over the study period ($r = -0.48$; $P < 0.001$), but the association from 14 August 2008 to 4 March 2009, when temperature decreased, had a stronger correlation ($r = -0.68$; $P < 0.00005$). After 11 March through 5 August 2009, when the temperature increased, there was substantially no relationship.

Other operating conditions, wastewater characteristics, and *G. amarae*-like cell concentrations. The data in Fig. 3b showed no significant difference in the overall average HRT (4.4 ± 0.2 h) from August 2008 to 2009 at the CWRP and over the time period

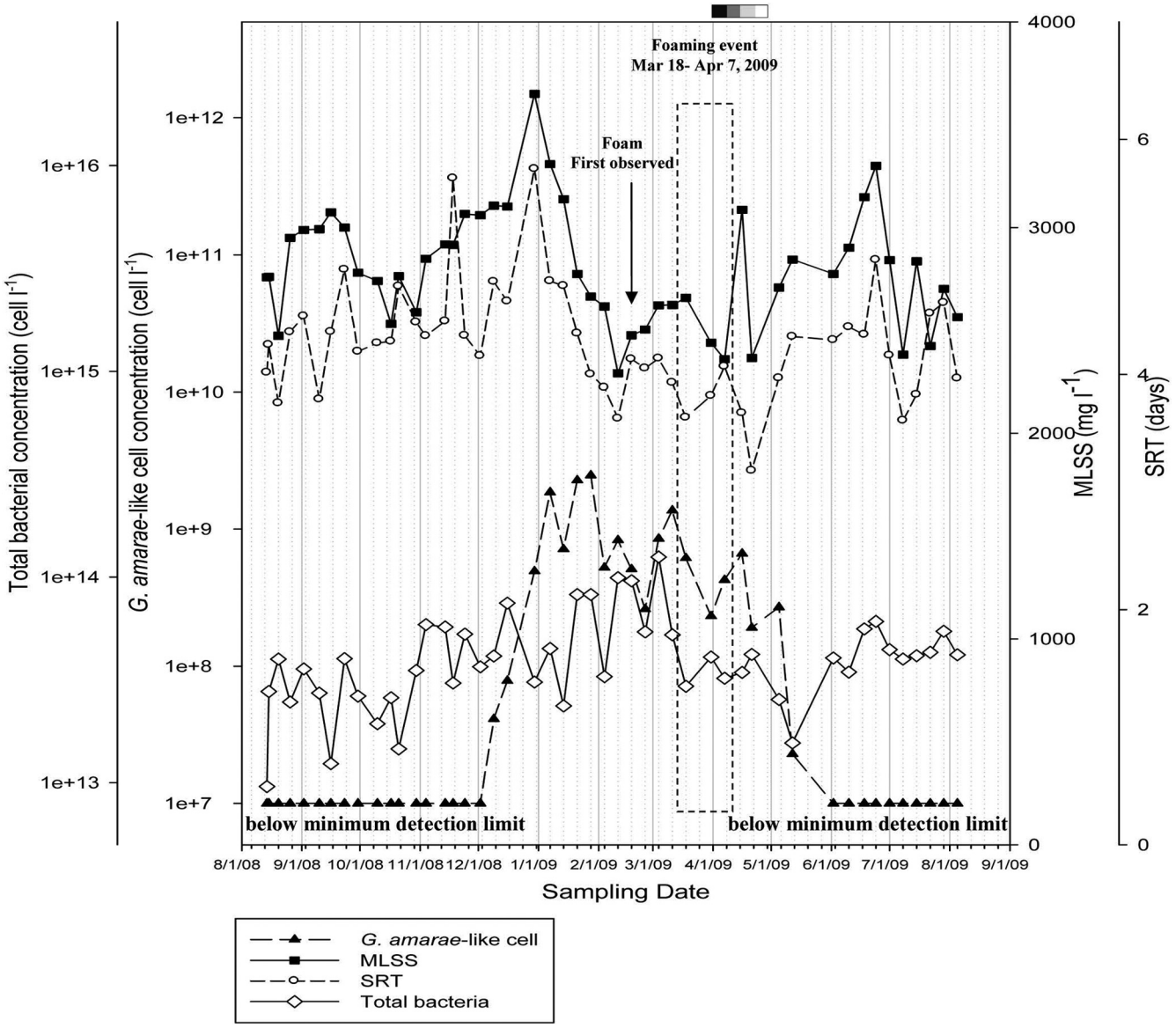

FIG 2 *G. amarae*-like cell concentrations (▲) (cells liter^{-1}), total bacterial concentrations in mixed liquor (◇) (cells liter^{-1}), SRT (○) (days), and MLSS (■) (mg liter^{-1}) at the CWRP over the study period (■, RAS chlorination; ■, SRT decrease; □, water spray and SRT decrease; □, RAS chlorination).

when the concentration of *G. amarae*-like bacteria exhibited an increase (HRT = 4.5 ± 0.1 h). However, if the HRT is examined over the period of this bacterium's rapid and late accelerating growth phases (9 December 2008 through 28 January 2009), there is a positive correlation of 0.79 with a P value of <0.05.

In Fig. 3b, the food-to-mass (F/M) ratio for the year is shown. The yearly average F/M ratio was 0.31 ± 0.07, while the average F/M ratio was 0.32 ± 0.06 when *G. amarae*-like cell concentrations were above the minimum detection limit. The F/M ratio had been low (0.18) on 2 December 2008, before *G. amarae*-like bacteria were first observed. During the period in which *G. amarae*-like bacteria were growing rapidly, from 2 to 16 December 2008, the F/M ratio increased from 0.18 to 0.53. An increase of the F/M ratio was also detected on 28 September 2008 (Fig. 3b), but the F/M ratio did not decrease prior to the increase on this date and did not impact *G. amarae*-like cell concentrations. Decreases followed by increases of the F/M ratio and BOD concentration in primary effluent occurred on 22 July 2009, and *Gordonia* spp. were detected by PCR-RLB using *Gordonia* genus-specific probes later that month (4). However, they were below the qPCR detection limit until the end of the study, on 5 August 2009.

The averages and standard deviations of DO concentrations for the entire study period were 0.87 ± 0.16 mg liter^{-1} and 0.99 ± 0.11 mg liter^{-1} during the time when *G. amarae*-like bacteria were observed (Fig. 3c). Higher DO concentrations occurred during cool weather. *G. amarae*-like cell and DO concentrations both increased during the accelerating growth phase and decreased during the washout phase.

The averages and standard deviations of ammonium ion concentrations in the primary effluent (NH_4^+-N) were 29.3 ± 5.4 mg liter^{-1} from August 2008 to 2009 and 25.6 ± 3.4 mg liter^{-1} during the time period when *G. amarae*-like bacteria were detected

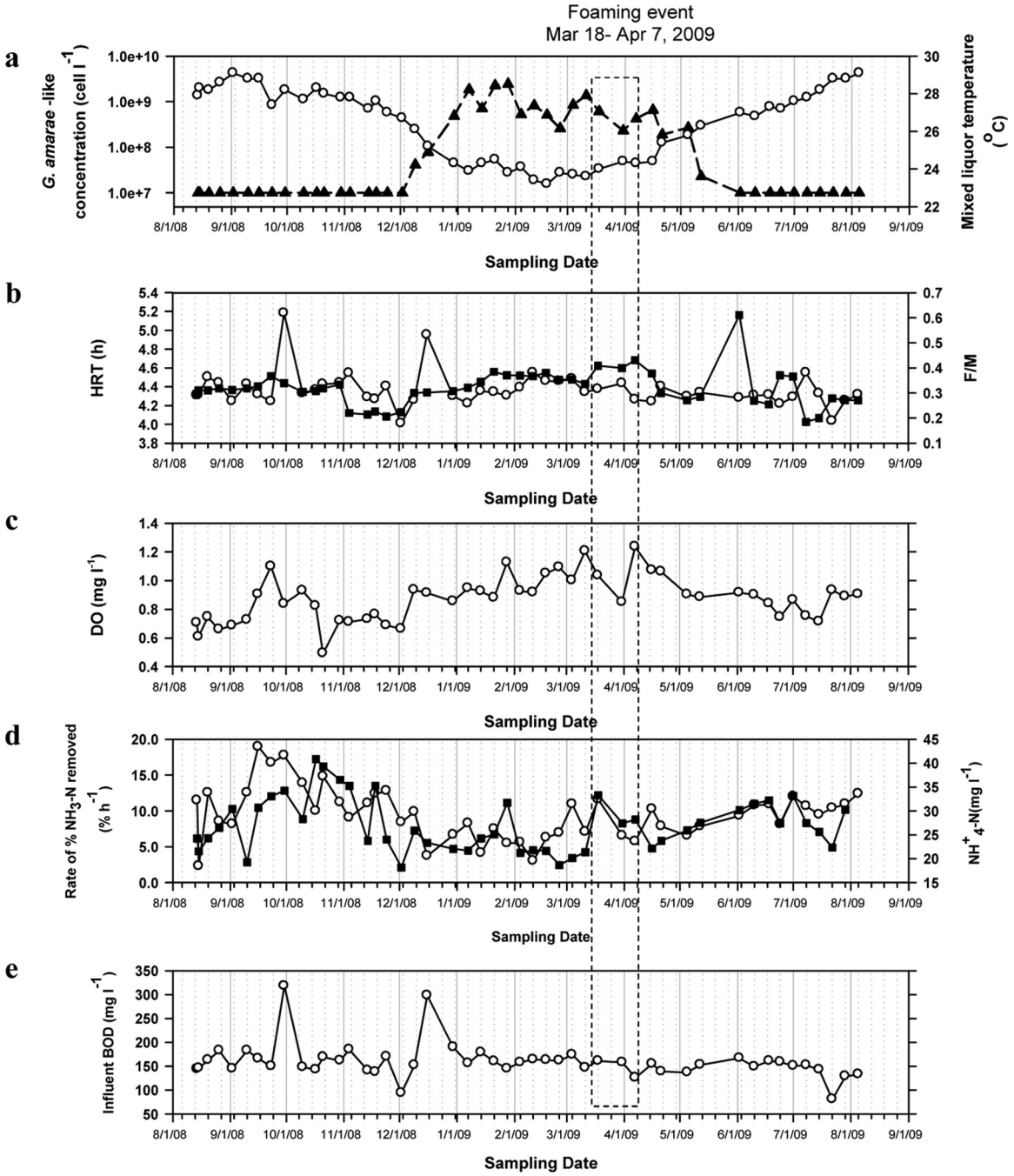

FIG 3 *G. amarae*-like cell concentration in mixed liquor with temperature (a), food-to-mass ratio with hydraulic retention time (b), dissolved oxygen concentration (c), ammonium ion concentration in primary effluent with percent ammonium ion removal rate (d), and BOD concentration in primary effluent (e) (▲, *G. amarae*-like cell concentration; ○, temperature, F/M ratio, and concentrations of DO, NH_4^+-N, and BOD; ■, percent NH_4^+-N removal rate and HRT).

(Fig. 3d). At the beginning of the *G. amarae*-like bacterial growth phase, the NH_4^+-N concentration in the primary effluent had a decreasing trend, but overall, no statistically significant relationship between this variable and *G. amarae*-like cell concentrations was shown. The percent NH_4^+-N removed per hour decreased during its rapid growth phase (9 December 2008 through 7 January 2009) and increased during the washout phase.

The averages and standard deviations of BOD concentrations in primary effluent from August 2008 to 2009 were 160 ± 38 mg liter^{-1} and 164 ± 35 mg liter^{-1} when *G. amarae*-like bacteria were detected (Fig. 3e). The BOD in primary effluent had a trend similar to that of the F/M ratio (Fig. 3b and e). When *G. amarae*-like cell concentrations were below the detection limit on 2 December 2008 and increased rapidly from 9 December 2008 through 7 January 2009, the BOD concentrations increased from 94 to 298 mg liter^{-1}. By 30 December 2008, the BOD concentrations had de-

creased to approximately 170 mg liter^{-1}, but *G. amarae*-like bacteria continued to grow, with no response to BOD concentrations in the primary effluent. BOD concentrations in the primary effluent remained at similar concentrations (Fig. 3e) throughout the remainder of the study.

DISCUSSION

Validation of the *Gordonia amarae* primer and probe set. Previous studies by de los Reyes et al. (17, 18) showed several differences among *Gordonia amarae* strains using both membrane hybridization and FISH. Although our primer and probe set was designed to be species specific, it appeared to detect substrain variation based on the sequencing results of the 159-bp fragments (see Fig. S2 in the supplemental material). This substrain variation produced sequencing results that did not match any known sequences of *G. amarae* in GenBank. In our study, the *G. amarae* primer and probe set was developed based upon a region of high homology within the 16S rRNA genes of *G. amarae* strains. Seven *G. amarae* 16S rRNA gene sequences in GenBank exhibited 95% to 100% homology with both primers. Additionally, G-amarae-Probe had a maximum of 3 mismatches in the probe region or 90 to 100% similarity to those seven *G. amarae* 16S rRNA gene sequences. A previous study by Xiao et al. (69), which differentiated species of *Nocardia*, showed that 5 mismatches were required before a probe would not adhere. This indicated that the 16S rRNA gene in the region of our target fragment is variable and emphasized the difficulty in the development of a *G. amarae*-specific primer and probe set and the plasticity of the rRNA.

Occurrence of *Gordonia amarae*-like and total bacterial cell concentrations. Different approaches are used to quantify concentrations of foaming nocardioforms in sewage treatment plants, but all of these, including determinations of intersections per volume, numerical rating using light microscopy, CFU, and optical density, do not address the growth phase (31, 32, 51). FISH evaluated the filamentous bacteria as length per volume or cells per volume, while hybridization methods report these bacteria as percent small-subunit (SSU) rRNA (11, 14). Our study is the first to use qPCR to quantify the accelerating growth, pseudo-steady-state, and washout phases of *G. amarae*-like bacteria (Fig. 2).

Although the exact triggering mechanisms of foaming remain to be elucidated, most studies have focused only on foaming events (19), while in this study, the growth cycle was examined, which allowed the analysis of potential triggering variables. The initial rapid growth (Fig. 2) in our study appeared to be similar to that described previously by de los Reyes and Raskin (14) using FISH, which showed that *Gordonia* spp. were first detected *in situ* in activated sludge samples as short single rods. Since foaming occurred approximately 40 days after *G. amarae*-like bacteria entered the pseudo-steady state, we hypothesize that branching occurred closer to the foaming period, which separates the early growth stage from the branched filamentous growth stage of *Gordonia* spp. and nocardioforms associated with foaming (33).

The foaming threshold of 2×10^9 mycolata cells liter^{-1} in the mixed liquor was proposed previously in the United Kingdom (12) for strongly hydrophobic genera of the mycolata group (12), while weakly hydrophobic mycolata at approximately 2×10^9 mycolata cells liter^{-1} with a 4.5-day sludge age did not cause foaming (11). Our study builds on those reported previously by Davenport et al. (11) and Oerther et al. (48), by showing that even with strongly hydrophobic filamentous bacteria like *Gordonia*, the concentration of 2×10^9 mycolata cells liter^{-1} in the mixed liquor did not produce the foam incident until 40 days after this concentration was reached. This finding is indirectly supported by recent work by Guo and Zhang (26) using pyrosequencing, which indicated that *G. amarae* cells were present in 100% of mixed liquor samples from the 14 plants tested, and many of these plants were not experiencing problems. This finding suggests that rapid growth precedes not only filament formation but also biosurfactant production and would explain why previous studies always observed an association between foaming incidents and filament length (28, 46). Our study also showed that once foam was visible on the surface, the eradication of *Gordonia*-like bacteria was very difficult, which suggests that interventions to eliminate the organism should occur at concentrations of 10^7 cells liter^{-1} in the mixed liquor.

Our findings provided a different insight into foaming, since increasing nocardioform lengths and percent SSU rRNA were positively correlated with the amount of foam on the surface of the aeration tanks (14, 46), while our data gave a strong warning signal of potential foaming long in advance of microscopic identification in March 2009, when foam was already at the surface. Hence, *G. amarae*-like bacteria should be easier to control using RAS chlorination during the single-cell stage, because the organism is confined primarily to the mixed liquor, as are bulking bacteria (5). The initiation of early detection would require monitoring to identify when growth begins with either qPCR or, possibly, FISH, if the single cell-stage can be observed easily in the floc.

Effect of SRT on *G. amarae*-like cell concentrations in mixed liquor. Increases of the MCRT or SRT have been frequently reported to be associated with foaming events (33) but cannot be used to predict them (9). Furthermore, a lagged SRT showed a much stronger relationship with *G. amarae*-like cell concentrations in the mixed liquor, because its growth rate was much lower than those of other bacterial groups in aeration tanks, as was reported previously (8, 60). This is the first paper to report the relationship of lagged SRT to the growth of foaming organisms. Weekly sampling was required to observe changes in the SRT that corresponded to initial increases in concentrations of *G. amarae*-like bacteria.

Short-term increases in the SRT during a critical time period, when other parameters also favored *G. amarae*-like bacterial growth, appeared to be a strong controlling variable. In this study, a 3-week increase in the SRT from 4.2 to 5.7 days (December 2008) (Fig. 2) provided sufficient time to accommodate the slower doubling time of *G. amarae*, 4 to 7 days, which corresponded with previously reported pure-culture studies (60). Although the SRT contributed to *G. amarae*-like bacterial growth during its accelerating growth phase (9 December 2008 through 7 January 2009), it did not seem to have an effect on when the foam was produced (Fig. 2).

Our findings are in agreement with those described previously by Oerther et al. (48), who observed that a long SRT was absent during a foaming incident in Illinois. Additionally, a long-term study of foaming at the same plant confirmed that the SRT was not the factor contributing to the occurrence of foam (23). Dhaliwal (20) also concluded that there was no relationship between foam and sludge age or the SRT at the plant studied, which was in agreement with our overall findings for the correlation between *G. amarae*-like bacterial concentrations and the SRT as well as between the foaming event and the SRT (Fig. 2).

A decrease of the SRT to less than 4.0 days, from 7 January to 18 March 2009, decreased *G. amarae*-like bacterial concentrations 1 order of magnitude (Fig. 2). At the same temperature (~24°C) as the one used in this study, Cha et al. (10) previously suggested that a 1.6-day MCRT or SRT could wash out nocardioform foaming. However, such a reduction during the winter slow-growth period for heterotrophic bacteria could cause a marked negative effect on floc size, because extracellular polymer formation by bacteria occurs 30 to 40 h after their maximum growth (49), and also settling at the CWRP, as reported previously (42). Hence, good floc formation requires an MCRT of approximately 3.3 days (49), making SRT control feasible only during initial growth.

Effect of mixed liquor temperature on *Gordonia amarae*-like cell concentrations. Many studies have shown temperature to be a significant parameter in determining bacterial growth in aeration tanks (13, 68). In our study, *G. amarae*-like bacterial growth increased when the temperature decreased (Fig. 3a); however, many reports on nocardioforms in mixed liquors and surface foams of aeration tanks at full-scale plants have associated growth with summer months (14, 55). A previous study of bench-scale activated sludge also found higher nocardioform filament counts at warmer temperatures when the MCRT was constant (10). The foaming episode in our study did occur as the temperature increased (late winter), which was agreement with previous studies from the Midwest, where filamentous bacterial growth and foaming occurred during late spring or summer, when temperatures increased 4°C to 6°C (14, 48).

Early studies found that foaming occurred during temperature shifts (14, 19), as in this study. Since different types of mycolata are selected for at different temperature ranges (5°C to 50°C), foaming may not be temperature restricted but rather triggered by changes in temperature (59). Our study found that temperature alone could not account for the increase in the *G. amarae*-like cell concentration and is supported by a previous study by Huang et al. (29) on nitrite-oxidizing bacteria, which could not separate temperature from the DO effect on population dynamics.

Relationships of other operating conditions and wastewater characteristics with *G. amarae*-like cell concentrations. In our study, decreasing followed by increasing F/M ratios and BOD concentrations in primary effluent were observed to be the most influential factors associated with the initial growth of *G. amarae*-like bacteria (Fig. 3b and e), while the HRT and the DO and NH_4^+-N concentrations in primary effluent did not display significant relationships with changes in *G. amarae*-like cell concentrations (Fig. 3b to d). Low F/M ratios were suggested previously to be an operational problem promoting nocardioforms in activated sludge (52) and were observed 1 week before *G. amarae*-like bacteria were detected (F/M ratio of 0.18). In July 2009, the F/M ratio and BOD concentration decreased and then increased, and *G. amarae*-like bacteria in the mixed liquor were detected by using PCR-RLB soon thereafter (4), but the qPCR portion of the study had ended, so this is not discussed further. We hypothesize that a drop in the BOD concentration or F/M ratio can trigger the initial growth of this foaming organism when other favorable conditions are present. However, extended monitoring periods across different plant types for the foaming organism during the early growth phase are required to draw any general conclusions.

Our findings fit with the strategy of nutrient switching (41). At a low F/M ratio, *G. amarae*-like bacteria appeared to act as *K* strategists (in which the organism utilizes a substrate for which it has a high substrate affinity or low saturation coefficient [K_s]), surviving on a restricted substrate by maintaining a minimal biomass. During periods of high F/M ratios, it switched to an *r* strategy, in which the intrinsic rate of the increase is equivalent to the μ_{max} (41), and the substrate concentration exceeds a critical value so that the K_s is no longer the controlling factor. The sawtooth patterns of total bacterial and *G. amarae*-like bacterial populations shown in Fig. 2 demonstrate multiple competing bacterial groups in the aeration tank (21), which was the result of the cumulative effects of shifting physicochemical parameters such as temperature, SRT, or substrate types or concentrations. Hence, different bacterial genera are favored as physicochemical parameters shift. The *G. amarae*-like cell concentrations in our study were maintained, although the F/M ratio returned to the average value (Fig. 3b). Nevertheless, no specific type or fraction of substrate can be postulated, since the extent of the analyses, as in most plants, was the measurement of NH_4^+-N and BOD concentrations.

Our data indicated that a cascade of events triggered the growth of *G. amarae*-like bacteria, involving substrate concentrations in combination with the SRT and temperature (Fig. 2 and 3b and e). This combination of factors could easily explain why single-variable analyses of produced foaming events provide contradictory results across plants (9).

The percent NH_4^+-N removed per hour decreased during rapid increases of *G. amarae*-like bacterial concentrations, and the percent NH_4^+-N removal increased during washout periods (Fig. 3d). Also, the inverse relationship between DO concentrations and the percent NH_4^+-N removal rate (Fig. 3c and d) may suggest that with a temperature drop, ammonia-oxidizing bacterial (AOB) activity decreased, resulting in less DO demand (24) and less ammonia removed over time. In this manner, the decreasing temperature acted to indirectly benefit *G. amarae*-like bacteria by making more DO available, as opposed to being a definitive controlling variable.

An understanding of the cause of *G. amarae* or nocardioform foaming events is complicated not only by the above-described points but also because the concentrations of organisms reported in the literature have been based on different methodologies (14, 20, 51). Foaming incidents have been presented as case studies, with many operational and environmental parameters being inadequately described (9, 20), or pure culture studies using controlled conditions and univariate analysis, which explain well multivariate interactions during full-scale plant operation. Additionally, foaming incidents described in the older literature may have been caused by different bacterial genera due to misidentification (56), further confounding the ability to determine the commonality of parameters surrounding foaming.

The present study demonstrated the value of *in situ* fine-scale sampling of actual plants over time periods when the water temperature changes. Fine-scale monitoring allowed an analysis of short-term changes in physicochemical variables that corresponded to *G. amarae*-like bacterial population dynamics during their increase, pseudo-steady state, or decline but not over the entire growth cycle. Sampling at 1-week intervals was inadequate over the 3- to 4-week time intervals of rapidly accelerating growth and washout phases to demonstrate significant correlations with *G. amarae*-like bacterial concentrations and variables such as the percent NH_4^+-N removal rate and DO concentration. Intense sampling during increases or decreases in concentrations could

better define key operational variables and identify triggering mechanisms of *G. amarae*-like bacterial growth and foam production.

To conclude, the interaction of wastewater characteristics and operational factors (temperature shifts, increased SRT, and low followed by high F/M ratios and BOD concentrations in primary effluent) and the ability of *G. amarae*-like bacteria to take advantage of favorable variations triggered the rapid growth of this organism. Quantitative assessments of *G. amarae*-like bacteria during initial increases in cell concentrations, when they are confined mainly to the floc, should increase the effectiveness of RAS chlorination alone or in combination with an increased wasting rate to eliminate the organism. Late detection, after filaments are found at the surface, made RAS chlorination or the SRT alone ineffective at ending the foaming event. Only after surface sprays resuspended this organism in the mixed liquor were these techniques successful.

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